Nrf2-Dependent Suppression of Azoxymethane/Dextran Sulfate Sodium–Induced Colon Carcinogenesis by the Cinnamon-Derived Dietary Factor Cinnamaldehyde

Min Long, Shasha Tao, Montserrat Rojo de la Vega, Tao Jiang, Qing Wen, Sophia L. Park, Donna D. Zhang, and Georg T. Wondrak

Abstract

The progressive nature of colorectal cancer and poor prognosis associated with the metastatic phase of the disease create an urgent need for the development of more efficacious strategies targeting colorectal carcinogenesis. Cumulative evidence suggests that the redox-sensitive transcription factor Nrf2 (nuclear factor-E2–related factor 2), a master regulator of the cellular antioxidant defence, represents a promising molecular target for colorectal cancer chemoprevention. Recently, we have identified cinnamon, the ground bark of *Cinnamomum aromaticum* (cassia cinnamon) and *Cinnamomum verum* (Ceylon cinnamon), as a rich dietary source of the Nrf2 inducer cinnamaldehyde (CA) eliciting the Nrf2-regulated antioxidant response in human epithelial colon cells, conferring cytoprotection against electrophilic and genotoxic insult. Here, we have explored the molecular mechanism underlying CA-induced Nrf2 activation in colorectal epithelial cells and have examined the chemopreventive potential of CA in a murine colorectal cancer model comparing Nrf2+/+ with Nrf2−/− mice. In HCT116 cells, CA caused a Keap1-C151–dependent increase in Nrf2 protein half-life via blockage of ubiquitination with upregulation of cytoprotective Nrf2 target genes and elevation of cellular glutathione. After optimizing colorectal Nrf2 activation and target gene expression by dietary CA-supplementation regimens, we demonstrated that CA suppresses AOM/DSS-induced inflammatory colon carcinogenesis with modulation of molecular markers of colorectal carcinogenesis. Dietary suppression of colorectal cancer using CA supplementation was achieved in Nrf2+/+ but not in Nrf2−/− mice confirming the Nrf2 dependence of CA-induced chemopreventive effects. Taken together, our data suggest feasibility of colorectal cancer suppression by dietary CA, an FDA-approved food additive derived from the third most consumed spice in the world.

Cancer Prev Res. 8(5): 444–54. © 2015 AACR.

Introduction

Colorectal cancer is a major cause of tumor-related morbidity and mortality worldwide (1, 2). Progression of colorectal cancer can occur over decades and involves the early development of adenomatous precursor lesions followed by invasive stages of the disease (3). Apart from other factors such as genetic predisposition involving somatic mutations of the tumor suppressor gene adenomatosis polyposis coli (APC), chronic inflammation with oxidative tissue damage is thought to contribute significantly to colorectal carcinogenesis (1, 2). Indeed, colorectal cancer risk is increased in the context of chronic inflammatory bowel disease, including Crohn’s disease and ulcerative colitis (4). The progressive nature of the disease and the poor prognosis associated with metastatic colorectal cancer create an urgent need for the development of more efficacious strategies targeting colorectal carcinogenesis, including chemoprevention (1, 2). Chemopreventive intervention aiming at pharmacologic suppression of colon carcinogenesis has shown promise in cellular and animal studies as well as human clinical trials (1, 5–7), but more efficacious and safer molecular agents are needed (8).

Recent research strongly suggests that the redox-sensitive transcription factor Nrf2 (nuclear factor-E2-related factor 2) is a promising molecular target for chemoprevention of colorectal carcinogenesis (9–13). Numerous natural products and dietary chemopreventive factors activate Nrf2 through covalent adduction and/or oxidation of redox-sensitive thiol residues in Keap1 (Kelch-like ECH-associated protein 1), the negative regulator of Nrf2 (14–17). Inhibition of Keap1-dependent ubiquitination and subsequent proteasomal degradation of Nrf2 allows nuclear translocation, a process followed by Nrf2-dependent transcriptional activation of cytoprotective target genes containing an antioxidant response element (ARE) regulatory sequence such as GCLC (glutamate-cysteine ligase catalytic subunit), GCLM (glutamate-cysteine ligase modifier subunit), TXN (thioredoxin), PRDX1 (peroxiredoxin 1), SRXN1 (sulfiredoxin 1), NQO1 [NAD(P)H quinone oxidoreductase 1], and HMOX1 (heme oxygenase-1; refs. 15, 16, 18). Consistent with its role as a master regulator of the cellular...
antioxidant stress response, Nrf2 has emerged as a critical factor determining susceptibility to inflammation-driven, colitis-associated colorectal cancer (9–11). Pharmacologic intervention using dietary factors that activate Nrf2 may represent a promising strategy for chemoprevention of cancer (17, 19, 20), and cumulative evidence obtained from animal studies strongly suggests feasibility of Nrf2-directed suppression of colorectal carcinogenesis (9, 12).

The ground bark of *Cinnamomum aromaticum* (cassia) and *Cinnamomum verum* (Ceylon cinnamon), commonly referred to as "cinnamon," together with pepper and vanilla is one of the three most consumed spices in the world (21), yet health effects of cinnamon consumption have remained mostly unexplored at the molecular level. Cinnamaldehyde (CA), the key flavor compound and predominant chemical constituent of cinnamon essential oil, is an FDA-approved flavorant and food additive with an established safety profile and extensive documentation of nontoxicity at FDA-approved oral doses (22–27). Recently, our detailed structure–activity relationship studies have identified CA as the key constituent in cinnamon powder responsible for potent induction of the Nrf2-regulated antioxidant response in human epithelial skin and colon cells, conferring cytoprotection against subsequent electrophilic, oxidative, and genotoxic insults (28, 29). Moreover, using Nrf2-wild-type versus Nrf2-knockout mice, the efficacy of CA-based therapeutic intervention targeting the progression of diabetic nephropathy through systemic Nrf2-activation was demonstrated (30).

Following our published prototype studies on CA as a potent Nrf2-inducer (28–30), we have now explored the chemopreventive potential of CA-dependent Nrf2 activation in a relevant murine model of colorectal cancer. After optimizing colorectal carcinogenesis in CA-supplemented Nrf2+/− mice.

Materials and Methods

Reagents

Cinnamaldehyde (CA) and azoxymethane (AOM) were purchased from Sigma-Aldrich. Dextran sulfate sodium (DSS) was obtained from Affymetrix Inc. Antibodies against Nrf2, NQO1, and ARNT were from Trevigen Inc. and Cayman Chemical, respectively. Anti-8-dihydro-2′-deoxyguanosine (8-oxo-dG) and anti-COX-2 antibodies were from Trevigen Inc. and Cayman Chemical, respectively.

Cell culture

Human colorectal carcinoma cells HCT116 obtained from the ATCC (CCL-247; authenticated by short-tandem repeat profiling and mycoplasma-tested by the vendor) were passaged for fewer than 6 months before experiments. Cells were maintained in DMEM (Cellgro) with 10% FBS (Cellgro) in a humidified incubator (37°C, 5% CO2).

Human Oxidative Stress RT²Profiler PCR Expression array analysis

Preparation of total cellular RNA, reverse transcription, and Human Oxidative Stress RT²Profiler PCR Expression Array (Super-Array) profiling was performed as described recently (31).

Transfection of cDNA and luciferase reporter gene assay

Transfection of cDNA was performed using Lipofectamine 2000 (Invitrogen) and used according to the manufacturer's instruction. Nrf2-dependent transcriptional activity was examined as previously published (32). HCT116 was transfected with an mGST-ARE firefly luciferase reporter plasmid together with expression plasmids for Nrf2, Keap1 (or Keap1C15S), and renilla luciferase (internal control). At 24-hour posttransfection, cells were treated with test compounds, and after 16 hours, cells were lysed for analysis of reporter gene activity. Reporter assays were performed using the dual-luciferase reporter gene assay system (Promega). For each experiment, samples were run in triplicate, and the data represent the mean of three independent repeats ± SD.

Immunoblot analysis, ubiquitination assay, and determination of protein half-life

Cells were harvested in sample buffer [50 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mmol/L DTT, and 0.1% bromophenol blue]. After sonication, cell lysates were electro-phoresed through an SDS-polyacrylamide gel and subjected to immunoblot analysis. For ubiquitination assay, cells were cotransfected with expression vectors for HA-tagged ubiquitin and Nrf2, and after 24 hours, cells were then treated with SF or CA (5 μmol/L, 4 hours) along with MG132 (10 μmol/L, ref. 33). Cells were then harvested in buffer [2% SDS, 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L DTT] and immediately boiled. The lysates were then diluted 5-fold in buffer lacking SDS and incubated with anti-Nrf2 antibody. Immunoprecipitated proteins were analyzed by immunoblotting using an antibody directed against the HA epitope (Santa Cruz Biotechnology). To measure Nrf2 protein half-life, cells were either left untreated or treated with CA (10 μmol/L, 4 hours), and cycloheximide (CHX, 50 μmol/L) was added to block protein synthesis. Total cell lysates were collected at different time points and subjected to immunoblot analysis with anti-Nrf2 antibody. The relative intensity of the bands was quantified using the ChemiDoc CRS gel documentation system and Quantity One software from Bio-Rad.

Glutathione assay

Total intracellular glutathione in cultured cells was analyzed using the luminescent GSH-Glo glutathione assay (Promega). Cells were harvested and then counted using a Z2 Coulter counter, and GSH was determined per 10,000 viable cells. Data represent relative levels of glutathione normalized for cell number comparing treated versus solvent controls (mean ± SD; n = 3).

Viability assay

Modulation of cell viability by exposure to CA was examined using flow-cytometric analysis of annexinV-FITC/propidium iodide (PI)-stained cells using an apoptosis detection kit according to the manufacturer’s specifications (APO-AF, Sigma) as published previously (28). Data indicate percentage viable (annexinV−/PI−) cells (comparing treated versus solvent controls; mean ± SD; n = 3).

Mouse experimentation and dietary supplementation

Experimental Nrf2+/+ and Nrf2−/− C57BL/6 mice were described previously (34). Mice were housed and handled in...
A

Gene symbol | Gene name | Fold change (untreated control)
---|---|---
HMOX1 | heme oxygenase (tyrosine) 1 | 124.67
SROX1 | sulfiredoxin 1 | 15.19
SLC7A11 | solute carrier family 7, member 11 | 15.35
AKR1C2 | aldo-keto reductase family 1, member C2 | 9.83
GCLM | glutamate-cysteine ligase, modifier subunit | 9.02
SQSTM1 | sequestosome 1 | 7.95
GCLC | glutamate-cysteine ligase, catalytic subunit | 7.24
FTH1 | ferritin, heavy polypeptide 1 | 6.86
THXR1 | thiosulfate oxidoreductase | 5.90
GPX3 | glutathione peroxidase 3 (gastrointestinal) | 5.17
GLA | galactose oxidase, alpha | 5.09
ALOX15 | arachidonate 15-lipoxygenase | 4.49
NQO1 | NAD(P)H dehydrogenase, quinone 1 | 4.26
GSR | glutathione reductase | 3.59
PTOR1 | prostaglandin reductase | 3.15
AGT1 | aldehyde oxidase 1 | 3.00

B

E

C

D

F

IP: | Nrf2 | IgG
---|---|---
SF | – | –
CA | – | –
tBHQ | – | +
Nrf2 | + | +
HA-Ub | + | +

IB: α-HA

Total Lysates | α-Nrf2 | α-Actin
---|---|---

G

Con | CA
---|---
0 | 1
15 | 30
45 | 60

Time (min)

Tublin

Chase time (min)

- Con $t_{1/2} = 17.6$ min
- CA $t_{1/2} = 40.7$ min

Con prevention research.

Published OnlineFirst February 23, 2015; DOI: 10.1158/1940-6207.CAPR-14-0359 Cancer Prevention Research
accordance with the Institutional Animal Care policies. Animals were maintained at 12-hour light/12-hour dark cycles with free access to water and standard diet (global 2913; Harlan Laboratories, Teklad diets). For dietary supplementation, CA was mixed with global 2919 diet, generating the desired final CA (0.1%, 0.5%; w/w) content. Preparation and replacement of CA-supplemented 2919 diet were performed daily. Absence of CA from un-supplemented control diet and stability of CA in the food matrix were monitored using our published GC-MS methodology confirming previous reports that document the overall chemical stability of CA used as a food additive as referenced in (28).

Colon epithelial Nrf2 upregulation by dietary CA supplementation

Eight-week-old mice (Nrf2+/−; 3 animals per group) were fed with standard diet (control) or received diet supplemented with CA (0.1% or 0.5%) for the duration of 5 days. On day six animals were sacrificed, and colon tissue was processed for analysis. The entire colon was excised, cut longitudinally, rinsed with ice-cold PBS, and fixed flat between sheets of filter paper. For immunoblot analysis, colon mucosa was scraped and stored at −80°C until use. For IHC analysis, colon tissue was fixed in 10% buffered formalin and paraffin embedded (12).

Dietary colon cancer chemoprevention using CA supplementation in AOM/DSS-exposed Nrf2−/− and Nrf2+/− mice

For dietary colon cancer chemoprevention using CA supplementation, colitis-associated carcinogenesis was modeled using an azoxymethane (AOM)/dextran sulfate sodium (DSS) exposure regimen according to a published standard procedure (35). Eight-week-old mice (Nrf2+/− vs. Nrf2−/−; 8–12 animals per group) were fed with standard diet or received diet supplemented with 0.5% CA. AOM injection (10 mg/kg, i.p.; 1 mg/mL in isotonic saline) was performed at the beginning of week 9, followed by three cycles of DSS administration (2.5% in drinking water; one week duration, each, followed by 2 weeks of regular water). CA supplementation was initiated one week before AOM injection and then maintained after AOM injection for one additional (CA2 AOM/DSS) or 10 weeks (CA1 AOM/DSS). Eleven weeks after the initiation of the experiment, mice were sacrificed for analysis.

Histopathological scoring system

Histopathological assessment of colon mucosa in Nrf2+/− and Nrf2−/− mice (6–12 specimens per treatment group) was performed at the end of the experiment. Histologic scores were assigned following the chronic colitis scoring system (36).

Hematoxylin and eosin staining and IHC

Tissue was stained using hematoxylin and eosin (H&E) according to standard procedures. Following antigen retrieval, tissue sections were incubated with 5% normal goat serum for 30 minutes followed by 16 hours incubation using the respective antibodies. Staining was performed using the Envision System HRP-DAB kit (DAKO) according to the manufacturer’s instructions. IHC staining was scored independently by two pathologists (intensity: four categories (from low to high): 0, 1, 2, and 3; percentage of positive cells: five categories: 0 (<10%), 1 (10%–25%), 2 (25%–50%), 3 (50%–75%), and 4 (75%–100%) as described recently (37). A final IHC staining score (intensity score × percentage score; 6–12 specimens per treatment group) was determined.

Statistics analysis

All results were analyzed using the GraphPad Prism software (version 6.0; GraphPad, CA; www.graphpad.com). Results obtained in vitro are presented as the mean ± SD of at least three independent experiments performed in duplicate or triplicate each. Data were analyzed using the two-sided Student’s t test, and selected datasets were analyzed using one-way ANOVA with Tukey’s post hoc test; means without a common letter differ. Where indicated, nonparametric data analysis of murine experimentation was performed using the Mann–Whitney test. Differences between groups were considered significant at P < 0.05.

Results

Cinnamaldehyde increases Nrf2 protein half-life via blockage of ubiquitination causing Nrf2 target gene expression and elevation of glutathione in HCT116 cells

First, to comprehensively monitor the antioxidant response gene expression upregulated by CA in HCT116 cells, oxidative

Figure 1. Molecular mechanism underlying CA modulation of Nrf2 activity in HCT116 cells. A, for Oxidative Stress RT2 Profiler PCR expression array analysis, cells were exposed to CA (20 μmol/L, 24 hours) followed by gene expression analysis. Top, scatter blot depiction of CA-induced gene expression (versus untreated); cut-off lines: 3-fold up- or downregulation; the insert shows the chemical structure of cinnamaldehyde; bottom, numerical expression changes (n = 3, mean ± SD; P < 0.05). B, upregulation of Nrf2 target gene-encoded proteins in response to CA exposure was examined in HCT116 cells treated with CA (1–20 μmol/L; 24 hours) using the luminescent GSH-Glo glutathione assay as detailed in Materials and Methods. Data are expressed as mean ± SD. Means without a common letter differ (ANOVA with Tukey post hoc test; *, P < 0.05; n = 3). C, CA modulation of ARE-luciferase reporter gene expression was assessed employing Kep1L-CIS5 and Kep1L-WT cotransfections. In addition, a Kep1L-siRNA against the 3′-untranslated region was cotransfected to suppress endogenous Kep1L. The transfected cells were then treated with CA (10 μmol/L), As(III) (10 μmol/L), SF (5 μmol/L), or tBHQ (50 μmol/L) for 16 hours, before the measurement of freely and Renilla luciferase activities. Data are expressed as mean ± SD (*, P < 0.05 control vs. compound treatment; #, P < 0.05 Kep1L-WT vs. Kep1L-CIS5). An aliquot of cell lysates was used for immunoblot analysis. F, CA modulation of Nrf2-ubiquitination was assessed by cotransflecting cells with plasmids encoding the indicated proteins (Nrf2, HA-Ub). Cells were then treated with SF, CA, or tBHQ (5 μmol/L; 4 hours) along with MG132 (10 μmol/L; 4 hours) before cell lysates were collected for the ubiquitination assay. For detection of ubiquitin-conjugated Nrf2, anti-Nrf2 immunoprecipitates were analyzed by immunoblotting using an anti-HA antibody. G, CA modulation of Nrf2 protein half-life. After cells were left untreated or treated with CA (10 μmol/L), cycloheximide (50 μmol/L) was added and cells were harvested at the indicated time points (0 to 60 minutes). Cell lysates were subjected to immunoblot analysis using anti-Nrf2 and anti-tubulin antibodies. Band intensity was quantified using Quantity One software and plotted against the time after cycloheximide treatment.
Finally, the molecular mechanism underlying CA-induced Nrf2 activation in HCT116 cells was explored. Using Keap1-C151S versus Keap1-wt cotransfection (in addition to Keap1-siRNA directed against the 3′-untranslated region to suppress endogenous Keap1; Fig. 1E, top) in an ARE-luciferase assay activity assay, it was observed that CA-induced Nrf2 activation depended on Keap1-C151. This observation is consistent with prior findings indicating that this cysteine residue represents an important redox sensor for CA-related electrophilic Michael acceptor pharmacophores, including 4-hydroxynonenal and acrolein (38). Similar results were obtained using other Keap1-Cys151–dependent Nrf2 inducers, including tBHQ and SF, whereas As (III) known to activate Nrf2 independently of Keap1-Cys151 served as a negative control (39). These results were also confirmed when CA modulation of Nrf2 protein levels was monitored by immunoblot analysis revealing that CA induction of Nrf2 was strongly attenuated in Keap1 C151S-transfectants (Fig. 1E; bottom panel). Next, since it has been demonstrated previously that established Nrf2 inducers such as SF and tBHQ cause Nrf2 activation through inhibition of the Keap1-mediated ubiquitination of Nrf2, a cellular ubiquitination assay was performed (Fig. 1F). HCT116 cells were first cotransfected with expression vectors for Nrf2 and hemagglutinin-tagged (HA)-ubiquitin and then exposed to Nrf2 activator test compound (CA, SF, tBHQ) or left untreated, followed by immunoprecipitation of Nrf2 and immunodetection of ubiquitinated protein. In cells treated with CA, ubiquitination of Nrf2 decreased dramatically compared with untreated control. Likewise, SF or tBHQ also decreased Nrf2 ubiquitination, consistent with the known attenuation of ubiquitination by these Nrf2 inducers (40, 41). Following these experiments, the half-life of endogenous Nrf2 protein and its modulation by CA exposure was measured in HCT116 cells (Fig. 1G). The half-life of Nrf2 underunteared conditions (control) was 17.6 minutes, whereas Nrf2 half-life more than doubled (40.7 minutes) in response to CA treatment (5 μmol/L).

Taken together, these results indicate that CA is a Keap1-C151–dependent Nrf2-activator that attenuates Nrf2 ubiquitination and stabilizes Nrf2 at the protein level upregulating antioxidant stress response gene expression.

Dietary CA supplementation elevates Nrf2 target gene expression in mouse colon epithelial cells

Next, we tested feasibility of Nrf2 induction in vivo examining the effects of CA dietary supplementation in murine colon epithelial cells (Fig. 2). Eight-week-old mice were fed with standard diet or received diet supplemented with 0.1% or 0.5% CA for 5 days followed by examination of Nrf2 and Nrf2 target gene expression at the protein level. This specific dose range was chosen based on prior murine studies examining toxicologic effects of chronic dietary CA supplementation [up to 33,000 ppm (i.e., 3.3%) in standard feed] administered over an extended period (between 3 months and 2 years) demonstrating the absence of carcinogenic effects at high dose levels (22–24). It was observed that 0.5% CA supplementation displayed superior efficacy causing upregulation of Nrf2 and Nrf2 target protein levels, including NQO1 (approximately 3-fold), γ-GCS (almost 5-fold), and others (AKR1B10, AKR1C1). Remarkably, even at lower doses (0.1%), CA supplementation displayed significant Nrf2-modulatory efficacy as confirmed by immunoblot (Fig. 2A) and IHC analysis (Fig. 2B). On the basis of the extensive Nrf2-enhancing effects observed with 0.5% CA supplementation that occurred in the absence of any adverse effects, this dose was chosen for the subsequent chemoprevention experiment. 

Dietary colon cancer chemoprevention using CA supplementation in AOM/DSS-exposed Nrf2<sup>+/+</sup> and Nrf2<sup>−/−</sup> mice

To explore the Nrf2-dependent chemopreventive potential of CA targeting colorectal cancer, we used a standard model of inflammatory carcinogenesis performed in Nrf2<sup>+/+</sup> and Nrf2<sup>−/−</sup> mice (Fig. 3). Eight-week-old mice (Nrf2<sup>+/+</sup> vs. Nrf2<sup>−/−</sup>; 8–12 animals per group) fed a standard diet or diet supplemented with 0.5% CA, received AOM injection and DSS administration in drinking water as specified (Fig. 3A). CA supplementation started one week before AOM injection and was then maintained for another week (2 weeks total, "CA2 AOM/DSS"). Because it has recently been demonstrated that after initiation has occurred Nrf2 upregulation may promote tumorigenic progression, we also included an additional prolonged feeding regimen to assess whether extended CA supplementation (10 more weeks after AOM, 11 weeks total; "CA11 AOM/DSS") may enhance tumorigenesis (16).

First, histopathological assessment of nontumorous colon mucosa in Nrf2<sup>+/+</sup> and Nrf2<sup>−/−</sup> mice at the end of the experiment was performed using the chronic colitis scoring system described in Materials and Methods (Fig. 3B and C). Remarkably, CA supplementation significantly attenuated histologic damage (with maintenance of crypt organization in normal mucosa) only in Nrf2<sup>−/−</sup> mice and decreased the chronic colitis histologic score (P < 0.05). Specifically, in colon tissue adjacent to tumors, histopathological changes were evident from epithelial erosion, loss of goblet cells, and massive lymphocytic infiltration, inflammatory alterations typical of AOM/DSS-induced damage that were suppressed by CA administration in Nrf2<sup>+/+</sup> but not in Nrf2<sup>−/−</sup> mice. Moreover, it was observed that AOM/DSS-induced weight loss was significantly attenuated by CA supplementation (P < 0.05), a
increase is significantly antagonized by dietary CA supplementation in Nrf2+/+ mice (Fig. 3E), whereas no such effect was observable in Nrf2−/− mice (Fig. 3F), demonstrating the Nrf2 dependence of CA antagonism of AOM/DSS-induced weight loss.

Next, it was observed that AOM/DSS-induced colon carcinogenesis was suppressed significantly by dietary CA supplementation in Nrf2+/+ but not in Nrf2−/− mice (Fig. 4 and Table 1). Remarkably, both CA supplementation regimens significantly suppressed tumor multiplicity by up to 45%, but no significant difference was observed between the "CA 2 AOM/DSS" and "CA11 AOM/DSS" treatment groups. Importantly, a chemopreventive effect of CA supplementation was observable only in Nrf2+/+ but not in Nrf2−/− mice demonstrating that CA-mediated protection is Nrf2-dependent. When stratified by tumor colonic location and size, cancer chemopreventive efficacy of CA was most pronounced suppressing the occurrence of proximal (irrespective of size) and large (>2 mm diameter) middle and distal colonic tumors (Table 1). Remarkably, in Nrf2+/+ mice, large tumors (>5 mm diameter) located in the middle section were observed exclusively in the "CA11" supplementation group, whereas none were detected in the "CA2" supplementation group (data not shown), an observation consistent with Nrf2-dependent enhancement of tumor growth once initiation has occurred (16). Moreover, pathologic grading of distal tumors (from Table 1) indicated that CA supplementation caused a significant change in adenoma/adenocarcinoma ratio per treatment group: In Nrf2+/+ mice, CA supplementation increased the adenoma/adenocarcinoma ratio from 1.25 (AOM/DSS only) to 2.6 (CA2) or 4.0 (CA11), suggesting a supplementation-induced attenuation of tumorigenic progression. This trend was not observed in Nrf2−/− mice where CA supplementation did not significantly change the adenoma/adenocarcinoma ratio observed in the "AOM/DSS only" group (1.3).

IHC analysis of molecular markers associated with tumorigenic progression and inflammatory tissue damage confirmed CA suppression of AOM/DSS-induced carcinogenesis (Fig. 5A). CA supplementation caused pronounced downregulation of Ki-67 (Fig. 5B), a nuclear proliferative factor known to be upregulated in colorectal cancer (42), and Ki-67–directed effects of the "CA11" supplementation regimen were more pronounced than "CA2"–induced changes. Likewise, attenuation of the expression levels of the polyamine-synthesis enzyme ornithine decarboxylase (ODC; Fig. 5C), and pronounced suppression of the AOM/DSS-induced inflammatory enzyme cyclooxygenase 2 (COX-2; Fig. 5D), important key factors in colorectal cancer progression, were observed in response to CA supplementation (5, 43). In addition, AOM/DSS-induced oxidative damage targeting nuclear DNA was attenuated by CA supplementation as indicated by reduced staining for 8-oxo-dG, an effect that was observed only in the "CA11" supplementation group (Fig. 5E). Interestingly, in the "AOM/DSS only" groups, IHC scores for COX-2 and 8-oxo-dG were elevated in Nrf2−/− versus Nrf2+/+ mice (Fig. 5D and E), consistent with an impaired antioxidant tissue defence due to the absence of functional Nrf2. Strikingly, as observed with CA effects on AOM/DSS-induced carcinogenesis, CA modulation of colorectal cancer progression markers was confined to Nrf2+/+ mice only (Fig. 5B–E), suggesting the strict Nrf2 dependence of the molecular effects of CA supplementation.
of additive contained abundantly in cinnamon, the ground bark (Ceylon cinnamon) and alternative medicine) dietary supplement (25 and cinnamon has recently emerged as a CAM (complementary
dence indicates a long history of use as a traditional medicine,
constituent of global importance, ethno-pharmacologic evi-
activities of cinnamon, but only a small number of mechanistic

Discussion
This preclinical prototype study has examined the feasibility of
dietary chemoprevention of colorectal cancer by the Nrf2
activator CA, a flavor compound and FDA-approved food
additive contained abundantly in cinnamon, the ground bark
of *Cinnamomum aromatica* (*cassia*) and *Cinnamomum verum*
(*Ceylon cinnamon*), the third most consumed spice in the world
after pepper and vanilla (21, 44). Apart from its role as a dietary
component, the third most consumed spice in the world
after pepper and vanilla (21, 44). Apart from its role as a dietary
component, the third most consumed spice in the world
after pepper and vanilla (21, 44). Apart from its role as a dietary

### AOM/DSS-induced body weight loss in Nrf2–/– and Nrf2+/+ mice

<table>
<thead>
<tr>
<th>Group</th>
<th>AOM/DSS</th>
<th>CA2 AOM/DSS</th>
<th>CA11 AOM/DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2–/–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nrf2+/+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Dietary CA supplementation decreases AOM/DSS-induced colitis in Nrf2–/– but not Nrf2+/+ mice. A, 8-week-old Nrf2–/– and Nrf2+/+ mice were fed with standard diet or received diet supplemented with 0.5% CA. AOM injection was performed at the beginning of week 9, followed by three cycles of DSS (2.5%) administration in drinking water. CA supplementation was initiated one week before AOM injection and then maintained after AOM injection for 1 (CA2) or 10 (CA11) weeks. Eleven weeks after the initiation of the experiment, mice were sacrificed for analysis. B, CA supplementation significantly decreased loss of crypts and maintained mucosal crypt organization in Nrf2–/– mice only, as demonstrated by H&E staining (×100 magnification). C, Histological assessment of colon mucosa in Nrf2–/– and Nrf2+/+ mice was performed following the chronic colitis scoring system described in Materials and Methods. Horizontal lines indicate median values (*, P < 0.05; Mann-Whitney test); N.S., not significant. D, AOM/DSS-induced weight loss was significantly attenuated by CA11 supplementation in Nrf2–/– mice. The graph indicates average body weight (mean ± SD) per treatment group at the beginning and at the end of the experimental regimen (11 weeks total duration; *, P < 0.05 vs. control; †, P < 0.05 versus AOM/DSS). E and F, AOM/DSS-induced body weight loss in Nrf2–/– versus Nrf2+/+ mice was analyzed as a function of CA supplementation. At the end of the treatment regimen, the relative weight loss was compared between treatment groups; the difference between AOM/DSS versus untreated control was numerically defined as 1 (†, P < 0.05).
Figure 4.
Dietary CA supplementation suppresses AOM/DSS-induced colon carcinogenesis in Nrf2<sup>+/+</sup> but not Nrf2<sup>−/−</sup> mice. At the end of the treatment regimen, total tumor number (distal, middle, proximal combined) per mouse was determined as a function of treatment regimen. Horizontal lines indicate median values for every treatment group. CA supplementation regimens was determined as a function of treatment regimen. Data indicate median tumor number per mouse (including range from minimum to maximum) as a function of size (diameter in mm) and anatomical location.

Table 1. Data indicate median tumor number per mouse (including range from minimum to maximum) as a function of size (diameter in mm) and anatomical location (distal, middle, proximal) per experimental group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nrf2&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Nrf2&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AOM/DSS</td>
<td>CA2 AOM/DSS</td>
</tr>
<tr>
<td>Distal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 mm</td>
<td>4.0 (2–7)</td>
<td>3.0 (3–5)</td>
</tr>
<tr>
<td>≥2 mm</td>
<td>3.0 (2–8)</td>
<td>1.0 (1–5)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Middle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 mm</td>
<td>2.0 (0–5)</td>
<td>2.0 (0–3)</td>
</tr>
<tr>
<td>≥2 mm</td>
<td>2.0 (0–3)</td>
<td>1.0 (0–2)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proximal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 mm</td>
<td>3.0 (0–4)</td>
<td>0.5 (0–3)</td>
</tr>
<tr>
<td>≥2 mm</td>
<td>1.0 (0–3)</td>
<td>0.0 (0–1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>14.5 (2–20)</td>
<td>10.0 (8–12)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05 compared with the Nrf2<sup>−/−</sup> AOM/DSS group; Mann–Whitney test.

The chemopreventive efficacy of Nrf2-activators administered orally has been demonstrated (12). In our murine model, a significant CA-dependent attenuation of AOM/DSS colon tumorigenesis was observed, and the crucial involvement of Nrf2-mediated mechanisms underlying CA-induced antitumorigenic effects was evident from the observation that CA chemopreventive effects were only detectable in Nrf2<sup>+/+</sup> mice (Figs. 3–5).

Earlier research has already generated strong genetic evidence supporting the protective role of Nrf2 against inflammatory colorectal carcinogenesis (9). Importantly, using Nrf2<sup>−/−</sup> versus Nrf2<sup>+/+</sup> mice, the crucial role of Nrf2 in determining susceptibility to inflammatory induction of colitis and colitis-driven colorectal carcinogenesis has been established (9–11, 13). Consistent with the antioxidant, anti-inflammatory, and cytoprotective cellular effects downstream of Nrf2 transcriptional activation, recent research demonstrated feasibility of chemoprevention targeting colitis-associated colorectal carcinogenesis in the murine AOM/DSS model by oral administration of experimental agents, including dibenzoylmethane, phenyl isothiocyanate, zernubene, and 2,3,4,4',5'-pentamethoxy-trans-stilbene known to induce Nrf2 activity (12, 48, 49).

In our search for potent and safe dietary Nrf2 activators, we recently focused on the cinnamon-derived dietary factor CA. Remarkably, CA is the only α,β-unsaturated aldehyde which is FDA approved for use in foods (21 CFR § 182.60) and given the “Generally Recognized As Safe” status by the “Flavor and Extract Manufacturers’ Association (FEMA)” in the United States (FEMA no. 2286; refs. 22–24, 50). Moreover, murine toxicity data, published in the context of safety profiling supporting FDA approval of CA as food additive, indicate nontoxicity of oral CA administration at supplementation levels as used in our pilot study (22–24, 44, 50). It is therefore reasonable to speculate that a potential chemopreventive use of this dietary factor contained in cinnamon powder may be achievable with an acceptable safety profile, an important prerequisite for the development of novel chemopreventive interventions administered to healthy or genetically predisposed cancer-prone individuals (8). CA content in food ranges from trace amounts (e.g., in orange juice) to 12.2 mg/100 g (122 ppm) in apple cinnamon cereals and 31.1 mg/100 g (311 ppm) for cinnamon swirl bread, suggesting that significant dietary intake of this potent Nrf2 inducer occurs in large populations (44). Future preclinical studies will aim at...
optimizing CA dosing regimens for dietary Nrf2 activation and will also test the feasibility of using the third most consumed spice in the world, cinnamon powder, as a widely accessible source of this unique and safe Nrf2 inducer for dietary cancer chemoprevention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Long, S. Tao, D.D. Zhang, G.T. Wondrak
Development of methodology: M. Long, S. Tao, Q. Wen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Long, M. Rojo de la Vega, Q. Wen, S.L. Park
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Long, M. Rojo de la Vega, T. Jiang, S.L. Park, D.D. Zhang, G.T. Wondrak
Writing, review, and/or revision of the manuscript: M. Long, M. Rojo de la Vega, D.D. Zhang, G.T. Wondrak

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Long, S. Tao, Q. Wen
Study supervision: D.D. Zhang, G.T. Wondrak

Grant Support
This work was supported in part by grants from the NIH (2R01ES015010 and R01CA154377, to D.D. Zhang; Arizona Cancer Center Support Grant CAD23074, ES0807091, ES06694, and R21CA166026, to G.T. Wondrak and D.D. Zhang), the State Scholarship Fund of China (201207610022, to M. Long), the National Natural Science Foundation of China (81228023, to D.D. Zhang), and Chongqing Science Foundation (cstc2013jcsfC10001-5, to M. Long).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 14, 2014; revised January 21, 2015; accepted February 9, 2015; published OnlineFirst February 23, 2015.
References

42. Zhang DD, Harnickin M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 2003;23:8137–51.

Nrf2-Directed Colon Cancer Chemoprevention by Cinnamaldehyde


Nrf2-Dependent Suppression of Azoxymethane/Dextran Sulfate Sodium–Induced Colon Carcinogenesis by the Cinnamon-Derived Dietary Factor Cinnamaldehyde

Min Long, Shasha Tao, Montserrat Rojo de la Vega, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-14-0359

Cited articles
This article cites 47 articles, 17 of which you can access for free at:
http://cancerpreventionresearch.aacrjournals.org/content/8/5/444.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/8/5/444.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.